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# PRIORITY DOCUMENT

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I, JULIE BILLINGSLEY, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. PS 3126 for a patent by JAMES COOK UNIVERSITY as filed on 21 June 2002.

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# PROVISIONAL SPECIFICATION

Invention Title: **Organ arrest, protection, preservation and recovery**

The invention is described in the following statement:

# ORGAN ARREST, PROTECTION, PRESERVATION AND RECOVERY

The present invention relates to a pharmaceutical composition for arresting, protecting and/or preserving an organ. The present invention also provides a method for arresting, protecting and preserving organs, in particular the heart during open-heart surgery, cardiovascular diagnosis or therapeutic intervention. The invention also relates to a method of recovering an organ from arrest, in particular after long-term arrest.

There are over 20,000 open-heart surgery operations each year in Australia, over 800,000 in the United States and about 1,000,000 in Europe. Of those requiring open-heart surgery, about 1.2% are neonates/infants primarily as a consequence of congenital heart disease.

The heart may be arrested for up to 3 hours during open-heart surgery. High potassium cardioplegia (in excess of 15-20 mM) has been the basis of myocardial arrest and protection for over 40 years. Currently the majority of solutions used contain high potassium including the widely used St Thomas No. 2 Hospital Solution which generally contains 110 mM NaCl, 16 mM KCl, 16 mM $MgCl_2$, 1.2 mM $CaCl_2$ and 10 mM $NaHCO_3$ and has a pH of about 7.8. High potassium solutions usually lead to a membrane depolarisation from about –80 to –50mV. Notwithstanding hyperkalemic solutions providing acceptable clinical outcomes, recent evidence suggests that progressive potassium induced depolarisation leads to ionic and metabolic imbalances that may be linked to myocardial stunning, ventricular arrhythmias, ischaemic injury, endothelial cell swelling, microvascular damage, cell death and loss of pump function during the reperfusion period. Infant hearts are even more prone to damage with cardioplegic arrest from high potassium than adult hearts. The major ion imbalances postulated are linked to an increased sodium influx which in turn activates the $Na^+/Ca^{2+}$ exchangers leading to a rise in intracellular $Ca^{2+}$. Compensatory activation of $Na^+$ and $Ca^{2+}$ ion pumps then occur, which activate anaerobic metabolism to replenish ATP with a concomitant increase in tissue lactate and fall in tissue pH. Free radical generation and oxidative stress have also been implicated in potassium arrest and partially reversed by the

administration of antioxidants. In some cases, high potassium induced ischaemia has been reported to have damaged smooth muscle and endothelial function.

In an attempt to minimise ischaemic damage during cardioplegic arrest, an increasing number of experimental studies have employed potassium channel openers instead of high potassium. Cardioprotection using nicorandil, aprikalim or pinacidil is believed to be linked to the opening of the potassium channel which leads to a hyperpolarised state, a shortening of the action potential and decreasing $Ca^{2+}$ influx into the cell. One shortfall however is that the heart takes the same time or longer to recover with no improvement in function than with high potassium cardioplegic solutions. Another limitation is that pinacidil requires a carrier due to its low solubility in aqueous solutions. The carrier routinely used is dimethyl sulphoxide (DMSO) which is controversial when used in animal or human therapy.

Most investigators, including those who advocate using potassium channel openers, believe that as soon as blood flow is halted and the arrest solution administered, ischaemia occurs and progressively increases with time. To reduce the likelihood of damage, the applicant sought a cardioplegic solution that would place the heart in a reversible hypometabolic state analogous to the tissues of a hibernating turtle, a hummingbird in torpor or an aestivating desert frog. When these animals drop their metabolic rate (some by over 90%), their tissues do not become progressively ischaemic but remain in a down-regulated steady state where supply and demand are matched. An ideal cardioplegic solution should produce a readily reversible, rapid electrochemical arrest with minimal tissue ischaemia. The heart should accumulate low tissue lactate, utilise little glycogen, show minimal changes in high-energy phosphates, cytosolic redox (NAD/NADH) and the bioenergetic phosphorylation (ATP/ADP Pi) ratio and free energy of ATP. There should be little or no change in cytosolic pH or free magnesium, minimal water shifts between the intracellular and extracellular phases, and no major ultrastructural damage to organelles such as the mitochondria. The ideal cardioplegic solution should produce 100% functional recovery with no ventricular arrhythmia, cytosolic calcium overload, or other pump abnormalities. There is no cardioplegic solution currently available which fulfils all these requirements.

The applicant previously found that the heart can be better protected by using the potassium channel opener adenosine and the local anaesthetic lignocaine to arrest and then preserve the heart (WO 00/56145), the entire disclosure of which is incorporated herein by reference.

The action of adenosine is controversial. Adenosine has been shown to increase coronary blood flow, hyperpolarise the cell membrane and act as a preconditioning agent via the ATP-sensitive potassium channel and adenosine related pathways including adenosine receptors notably the A1 receptor. Other receptors include $A2_a$ $A2_b$ and A3 receptors. Adenosine is also known to improve myocardial recovery as an adjunct to high potassium cardioplegia. Furthermore, adenosine can be used as a pretreatment (whether or not it is present in the arresting solution) to reduce lethal injury. In one study, adenosine was shown to rival potassium arrest solutions and more recently in blood cardioplegia, it prevented post-ischaemic dysfunction in ischaemically injured hearts. Adenosine is sometimes added as an adjunct to potassium cardioplegia.

Lignocaine is a local anaesthetic which blocks sodium fast channels and has antiarrhythmatic properties by reducing the magnitude of inward sodium current. The accompanying shortening of the action potential is thought to directly reduce calcium entry into the cell via $Ca^{2+}$ selective channels and $Na^{+}/Ca^{2+}$ exchange. Recent reports also implicate lignocaine with the scavenging of free radicals such as hydroxyl and singlet oxygen in the heart during reperfusion. Associated with this scavening function, lignocaine may also inhibit phospholipase activity and minimise membrane degradation during ischaemia. Lignocaine has also been shown to have a myocardial protective effect and in one study was found to be superior to high potassium solutions. However, our experiments show that lignocaine alone at 0.5, 1.0 and 1.5 mM gave highly variable functional recoveries using the isolated working rat heart.

Accordingly, the combination of the potassium channel opener and local anaesthetic result in arrest and better protection of the organ under normal potassium concentration (ie, physiological levels of potassium), thus reducing the risk of potassium induced injury to the organ which prior art high potassium arrest

solutions may induce. This solution containing these components was shown by the applicant to improve functional recovery from arrest of the organ over existing solutions.

However, although this solution provides improved recovery of the arrested organ over relatively short periods, there is still a need for a composition for arresting then preserving an organ for extended periods (long-term arrest), for example, beyond 4 to 6 hours. This would be particularly advantageous, for example, for transplanting organs which have been removed from a first patient and to be transplanted into a second patient (or recipient), located at a distance from each other which may prevent transplantation using currently available arrest solutions. There is also a need for a composition providing improved preservation and faster recovery of organs, which are otherwise difficult to recover from surgery after arrest, for example neonate/infant hearts.

It is therefore an object of this invention to overcome or at least alleviate, one or more of the difficulties or deficiencies associated with the prior art.

In particular, it is an object of this invention to provide a pharmaceutical composition for arresting, protecting and/or preserving an organ, with improved recovery of an organ, after arrest of the organ. It is also an object of this invention to provide a method for arresting, protecting and/or preserving an organ.

Accordingly, in one aspect of the present invention there is provided a pharmaceutical composition for arresting, protecting and/or preserving an organ including an effective amount of:

a primary potassium channel opener or agonist and/or adenosine receptor agonist;

a local anaesthetic; and

an impermeant.

The applicant has surprisingly found that the pharmaceutical composition of the present invention protects and preserves the organ after arrest of the organ, particularly after long-term arrest, with good to excellent recoveries of function after reperfusion.

The potassium channel openers or agonists may be selected from the group consisting of: nicorandil, diazoxide, minoxidil, pinicadil, aprikalim, cromokulim, NS-1619 (1,3-dihydro-1-[2-hydroxy5(trifluoromethyl)phenyl]5-(trifluoromethyl)2-H-benimidazol-one), amlodipine, Bay K 8644(L-type)(1,4-dihydro-26-dimethyl-5-nitro-4[2(trifluoromethyl)phenyl]-3-pyridine carboxylic acid (methyl ester)), bepridil HCl (L-type), calciseptine (L-type), omega-conotoxin GVIA (N-type), omega-conotoxin MVIIC (Q-type), cyproheptadine HCl, dantrolene sodium ($Ca^{2+}$ release inhibitor), diltiazem HCl (L-type), filodipine, flunarizine HCl ($Ca^{2+}$/$Na^{+}$), fluspirilene (L-type), HA-1077 2HCl(1-(5 isoquinolinyl sulphonyl) homo piperazine.HCl), isradipine, loperamide HCl, manoalide ($Ca^{2+}$ release inhibitor), nicardipine HCl (L-type), nifedipine (L-type), niguldipine HCl (L-type), nimodipine (L-type), nitrendipine (L-type), pimozide (L- and T-type), ruthenium red, ryanodine (SR channels), taicatoxin, verapamil HCl (L-type), methoxy-verapamil HCl (L-type), YS-035 HCl (L-type)N[2(3,4-dimethoxyphenyl)ethyl]-3,4-dimethoxy N-nethyl benzene ethaneamine HCl) and AV blockers such as verapamil and adenosine. It will be appreciated that this list includes calcium antagonists as potassium channel openers are indirect calcium antagonists.

Adenosine is particularly preferred as the primary potassium channel opener or agonist. Adenosine is capable of opening the potassium channel, hyperpolarising the cell, depressing metabolic function, possibly protecting endothelial cells, enhancing preconditioning of tissue and protecting from ischaemia or damage. Adenosine is also an indirect calcium antagonist, vasodilator, antiarrhythmic, antiadrenergic, free radical scavenger, arresting agent, anti-inflammatory agent (attenuates neutrophil activation), metabolic agent and possible nitric oxide donor.

Suitable adenosine receptor agonists may be selected from: $N^6$-cyclopentyladenosine (CPA), N-ethylcarboxamido adenosine (NECA), 2-[p-(2-

carboxyethyl)phenethyl-amino-5'-N-ethylcarboxamido adenosine (CGS-21680), 2-chloroadenosine, $N^6$-[2-(3,5-demethoxyphenyl)-2-(2-methoxyphenyl]ethyladenosine, 2-chloro-$N^6$-cyclopentyladenosine (CCPA), N-(4-aminobenzyl)-9-[5-(methylcarbonyl)-beta-D-robofuranosyl]-adenine (AB-MECA), ([IS-[1a,2b,3b,4a(S*)]]-4-[7-[[2-(3-chloro-2-thienyl)-1-methyl-propyl]amino]-3H-imidazole[4,5-b]pyridyl-3-yl]cyclopentane carboxamide (AMP579), $N^6$-(R)-phenylisopropyladenosine (R-PLA), aminophenylethyladenosine 9APNEA) and cyclohexyladenosine (CHA).

In a preferred embodiment, the pharmaceutical composition according to the present invention further includes a secondary potassium channel opener or agonist. The secondary potassium channel opener or agonist may provide additional cellular protection.

Preferably the secondary potassium channel opener is a mitochondrial ATP-sensitive potassium channel opener. More preferably, the mitochondrial ATP-sensitive potassium channel opener is diazoxide. Diazoxide is believed to preserve ion and volume regulation, oxidative phosphorylation and mitochonrial membrane integrity (appears concentration dependent). More recently, diazoxide affords cardioprotection by reducing mitochonrial oxidant stress at reoxygenation. At present it is not known if the protective effects of potassium channel openers are associated with modulation of reactive oxygen species generation in mitochondria.

The local anaesthetic component of the pharmaceutical composition according to the present invention may be selected from mexiletine, diphenylhydantoin prilocaine, procaine, mepivacaine and Class 1B antiarrhythmic agents such as lignocaine or derivatives thereof, for example, QX-314.

Preferably the local anaesthetic is Lignocaine. Lignocaine is preferred as it is capable of acting as a local anaesthetic probably by blocking sodium fast channels, depressing metabolic function, lowering free cytosolic calcium, protecting against enzyme release from cells, possibly protecting endothelial cells

and protecting against myofilament damage. Lignocaine is also a free radical scavenger and an antiarrhythmic.

As lignocaine acts by blocking sodium fast channels, it will be appreciated that other sodium channel blockers could be used instead of or in combination with the local anaesthetic in the method and composition of the present invention. Examples of suitable sodium channel blockers include venoms such as tetrodotoxin.

The impermeant component of the pharmaceutical composition according to the present invention may be selected from one or more of the group consisting of: sucrose, pentastarch, hydroxyethyl starch, raffinose, mannitol, gluconate, lactobionate, polyethylene glycol (PEG).

Preferably, the impermeant is sucrose. Sucrose reduces water shifts as an impermeant.

In another embodiment of the present invention there is provided a pharmaceutical composition according to the present invention, further including an effective amount of an antioxidant.

The antioxidant component of the pharmaceutical composition according to the present invention may be selected from one or more of the group consisting of: allopurinol, carnosine, Coenzyme Q 10, n-acetyl-cysteine, superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GP), catalase and the other metalloenzymes, glutathione, U-74006F, vitamin E, Trolox (soluble form of vitamin E), Vitamin C, Beta-Carotene (plant form of vitamin A), selenium, Gamma Linoleic Acid (GLA), alpha-lipoic acid, uric acid (urate), curcumin, bilirubin, proanthocyanidins, epigallocatechin gallate, Lutein, lycopene, bioflavonoids and polyphenols.

Preferably, the antioxidant is allopurinol. Allopurinol is a competitive inhibitor of the reactive oxygen species generating enzyme xanthine oxidase. Allopurinol's antioxidative properties may help preserve myocardial and

endothelial functions by reducing oxidative stress, mitochondrial damage, apoptosis and cell death.

In another embodiment of the present invention there is provided a pharmaceutical composition according to the present invention, further including an effective amount of a sodium hydrogen exchange inhibitor. The sodium hydrogen exchange inhibitor reduces sodium and calcium entering the cell.

The sodium hydrogen exchange inhibitor may be selected from one or more of the group consisting of amiloride, cariporide, eniporide, triamterene and EMD 84021, EMD 94309, EMD 96785 and HOE 642 and T-162559 are other inhibitors of the isoform 1 of the $Na^+/H^+$ exchanger.

Preferably, the sodium hydrogen exchange inhibitor is Amiloride. Amiloride inhibits the sodium proton exchanger ($Na^+/H^+$ exchanger also often abbreviated NHE-1) and reduces calcium entering the cell. During ischemia excess cell protons (or hydrogen ions) are exchanged for sodium via the $Na^+/H^+$ exchanger.

In yet another embodiment of the present invention there is provided a pharmaceutical composition according to the present invention, further including an effective amount of:

a source of magnesium; and

a source of calcium,

such that the pharmaceutical composition exhibits relatively high magnesium and low calcium concentrations.

Elevated magnesium and low calcium has been associated with protection during ischemia and reoxygenation of the organ. The action is believed due to decreased calcium loading.

Preferably the magnesium is present at a concentration of between 0.5mM to 20mM, more preferably about 2.5mM. Preferably the calcium present is at a concentration of between 0.1mM to 2.5mM, more preferably about 0.3mM.

In another preferred embodiment of the present invention, there is provided a pharmaceutical composition according to the present invention including an effective amount of:

a primary potassium channel opener or agonist and/or adenosine receptor agonist;

a local anaesthetic; and

an impermeant,

and further including an effective amount of one or more components selected from:

a secondary potassium channel opener or agonist;

an antioxidant;

a sodium hydrogen exchange inhibitor;

a source of magnesium; and

a source of calcium.

The term "organ" is used herein in its broadest sense and refers to any part of the body exercising a specific function including tissues and cells or parts thereof, for example, cell lines or organelle preparations. Other examples include circulatory organs such as the heart, respiratory organs such as the lungs, urinary organs such as the kidneys or bladder, digestive organs such as the stomach, liver, pancreas or spleen, reproductive organs such as the scrotum, testis, ovaries or uterus, neurological organs such as the brain, germ cells such as spermatozoa

or ovum and somatic cells such as skin cells, heart cells ie, myocytes, nerve cells, brain cells or kidney cells.

The pharmaceutical composition according to the present invention is highly beneficial at about 10°C, where longer arrest times using St Thomas No. 2 solution may only be achieved when the temperature is lowered, for example, down to about 4°C.

The pharmaceutical composition of the present invention is particularly useful in arresting, protecting and/or preserving the heart during open-heart surgery including heart transplants. Other applications include reducing heart damage before, during or following cardiovascular intervention which may include a heart attack, angioplasty or angiography. For example, the composition could be administered to subjects who have suffered or are developing a heart attack and used at the time of administration of blood clot-busting drugs such as streptokinase. As the clot is dissolved, the presence of the composition may protect the heart from further injury such as reperfusion injury. The pharmaceutical composition may be particularly effective as a cardioprotectant in those portions of the heart that have been starved of normal flow, nutrients and/or oxygen for different periods of time. For example, the pharmaceutical composition may be used to treat heart ischaemia which could be pre-existing or induced by cardiovascular intervention.

Accordingly, the present invention also provides a cardioplegic and/or cardioprotectant composition including an effective amount of:

a primary potassium channel opener or agonist and/or adenosine receptor agonist;

a local anaesthetic; and

an impermeant.

In a preferred embodiment of this aspect of the invention, the cardioplegic and/or cardioprotectant composition further includes a secondary potassium channel opener or agonist.

More preferably, the cardioplegic and/or cardioprotectant composition further includes an effective amount of one or more components selected from:

an antioxidant;

a sodium hydrogen exchange inhibitor;

a source of magnesium; and

a source of calcium.

According to another aspect of the present invention there is provided use of the pharmaceutical composition according to the present invention in the manufacture of a medicament for arresting, protecting and/or preserving an organ.

In a preferred embodiment of this aspect of the present invention it is preferred to aerate the pharmaceutical composition with a source of oxygen before and/or during use. The source of oxygen may be an oxygen gas mixture where oxygen is the predominant component. The oxygen may be mixed with, for example $CO_2$. Preferably, the oxygen gas mixture is 95% $O_2$ and 5% $CO_2$.

It is considered that the oxygenation with the oxygen gas mixture maintains mitochondrial oxidation and this helps preserve the myocyte and endothelium of the organ.

In another aspect of the present invention there is provided a method for arresting, protecting and/or preserving an organ including:

providing a pharmaceutical composition including an effective amount of

a primary potassium channel opener or agonist and/or adenosine receptor agonist and a local anaesthetic in a suitable container; and

a source of oxygen;

aerating the pharmaceutical composition with the oxygen; and

placing the organ in contact with the pharmaceutical composition under conditions sufficient to arrest, protect and/or preserve thereof.

Preferably the oxygen source is an oxygen gas mixture. Preferably oxygen is the predominant component. The oxygen may be mixed with, for example $CO_2$. More preferably, the oxygen gas mixture is 95% $O_2$ and 5% $CO_2$.

Preferably the pharmaceutical composition is aerated before and/or during contact with the organ.

Preferably the pharmaceutical composition according to this aspect of the invention is in liquid form. Liquid preparations of the pharmaceutical composition may take the form of, for example, solutions, syrups, or suspensions, or may be presented as a dry product for constitution with water or other suitable vehicle. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents, emulsifying agents, non-aqueous vehicles, preservatives and energy sources.

According to this aspect of the present invention there is provided a method for arresting, protecting and/or preserving an organ, wherein the pharmaceutical composition further includes an effective amount of one or more components selected from:

a secondary potassium channel opener or agonist;

an impermeant;

an antioxidant;

a sodium hydrogen exchange inhibitor;

a magnesium source; and

a calcium source.

While the present invention is particularly advantageous in arresting, protecting and/or preserving an organ while intact in the body of a subject, for example in the treatment of the heart in circumstances of myocardial infarction or heart attack, it will also be appreciated that the present invention may also be used to arrest, protect and/or preserve isolated organs.

The subject may be a human or an animal such as a livestock animal (eg, sheep, cow or horse), laboratory test animal (eg, mouse, rabbit or guinea pig) or a companion animal (eg, dog or cat), particularly an animal of economic importance.

The method of the present invention involves contacting an organ with the pharmaceutical composition, for a time and under conditions sufficient for the organ to be arrested, protected and/or preserved.

While it is possible for each component of the pharmaceutical composition to contact the organ alone, it is preferable that the components of the pharmaceutical composition be provided together with one or more pharmaceutically acceptable carriers, diluents, adjuvants and/or excipients. Each carrier, diluent, adjuvant and/or excipient must pharmaceutically acceptable such that they are compatible with the components of the pharmaceutical composition and not harmful to the subject. Preferably, the pharmaceutical composition is prepared with liquid carriers, diluents, adjuvants and/or excipients.

Accordingly, this aspect of the invention also provides a method for arresting, protecting and/or preserving an organ, which includes providing the pharmaceutical composition together with a pharmaceutically acceptable carrier, diluent, adjuvant and/or excipient.

A preferred pharmaceutically acceptable carrier is a buffer having a pH of about 6 to about 9, preferably about 7, more preferably about 7.4 and/or low concentrations of potassium, for example, up to about 10mM, more preferably about 2 to about 8 mM, most preferably about 4 to about 6mM. Suitable buffers include Krebs-Henseleit which generally contains 10mM glucose, 117 mM NaCl, 5.9 mM KCl, 25 mM $NaHCO_3$, 1.2 mM $NaH_2PO_4$, 1.12 mM$CaCl_2$ (free $Ca^{2+}$=1.07mM) and 0.512 mM $MgCl_2$ (free $Mg^{2+}$=0.5mM), St. Thomas No. 2 solution, Tyrodes solution which generally contains 10mM glucose, 126 mM NaCl, 5.4 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.33 mM $NaH_2PO_4$ and 10 mM HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethane sulphonic acid], Fremes solution, Hartmanns solution which generally contains 129 NaCl, 5 mM KCl, 2 mM $CaCl_2$ and 29 mM lactate and Ringers-Lactate. One advantage of using low potassium is that it renders the present composition less injurious to the subject, in particular pediatric subjects such as neonates/infants. High potassium has been linked to an accumulation of calcium which may be associated with irregular heart beats during recovery, heart damage and cell swelling. Neonates/infants are even more susceptible than adults to high potassium damage during cardiac arrest. After surgery for defects a neonate/infant's heart may not return to normal for many days, sometimes requiring intensive therapy or life support. It is also advantageous to use carriers having low concentrations of magnesium, such as, for example up to about 2.5mM, but it will be appreciated that high concentrations of magnesium, for example up to about 20mM, can be used if desired without substantially affecting the activity of the composition.

In another embodiment of the present invention there is provided use of a pharmaceutical composition for arresting, protecting and/or preserving an organ including an effective amount of:

a potassium channel opener or agonist and/or adenosine receptor agonist and;

a local anaesthetic;

provided in a suitable container together with a source of oxygen;

wherein the pharmaceutical composition is aerated with the oxygen and contacts the organ.

Preferably the oxygen source is an oxygen gas mixture. Preferably, oxygen is the predominant component. The oxygen may be mixed with for example $CO_2$. More preferably, the oxygen gas mixture is 95% $O_2$ and 5% $CO_2$.

Preferably the pharmaceutical composition is aerated before and/or during contact with the organ.

In another aspect of this embodiment of the invention, there is provided a system for arresting, protecting and/or preserving an organ, including a pharmaceutical composition including an effective amount of:

a potassium channel opener or agonist and/or adenosine receptor agonist; and

a local anaesthetic;

the pharmaceutical composition further including effective amounts of one or more components selected from:

a mitochondrial potassium channel opener or agonist;

an impermeant;

an antioxidant;

a sodium hydrogen exchange inhibitor;

a magnesium; and

a calcium source,

in combination with a source of oxygen.

In another preferred embodiment of the present invention there is also provided a reperfusion solution which is administered after long-term arrest protection and preservation with the solution according to the invention.

Preferably, the reperfusion solution is Krebs Henseleit buffer. More preferably, the Krebs Henseleit buffer includes an effective amount of one or more components selected from :

an impermeant;

a magnesium and a calcium source;

an antioxidant;

a sodium hydrogen exchange inhibitor; and

an energy substrate.

Preferably, the reperfusion solution is provided at 37°C.

The energy substrate helps with recovering metabolism. The energy substrate can be selected from one or more components selected from the group consisting of: pyruvate, glutamate, aspartate, arginine, lactate, glucose, insulin, alpha-keto glutarate, malate, succinate, carnitine.

The invention will now be described with reference to the following examples. These examples are not to be construed as limiting in any way.

## EXAMPLES

### Materials and Methods

**Animals and Surgical Procedures**: Male Sprague-Dawley rats weighing 300-400 g were obtained from Monash University and housed in the animal facility at James Cook University. Animals will have continual access to food and water. Rats anaesthetised with an intraperitoneal injection of sodium pentobarbital (60-70

mg/kg) and the heart excised and placed in cold Krebs Henseleit. All enzymes, chemicals and compounds were obtained from Sigma or Boehringer-Mannheim. Lignocaine were purchased from the local organ arrest, protection and/or preservation suppliers. Adenosine was purchased from Biomedical Res. Ltd (Sigma).

**Isolated Working Rat Heart**: Hearts were perfused in the working mode. Oxygen and $CO_2$ tensions, and pH in the arterial and venous perfusion lines were measured using a Corning 865 pH/blood gas-ion-analyser. Physiological variables were measured using a single channel Mac-Lab with a pressure transducer (UFI-1050) attached.

**Preservation Solution:** 200 uM adenosine plus 0.5 mM lignocaine, 10 uM diazoxide, 70 mM sucrose, 100 uM allopurinol in Krebs Henseleit (described below) and 10 mM glucose (gently aerated with 95% $O_2$ and 5% $CO_2$). The pH of the solution at 10°C was approximately 7.3, $pCO_2$ = 53 mmHg and $pO_2$ around 700 mmHg $O_2$. Note: $CaCl_2$ is 0.3 mM and $MgCl_2$ is 2.5 mM.

**Krebs-Henseleit buffer**: NaCl (117 mM), KCl (5.9 mM), $NaHCO_3$ (25 mM), $NaH_2PO_4$, (1.2 mM) 1.12 mM $CaCl_2$ (free $Ca^{2+}$ = 1.07 mM), 0.512 mM $MgCl_2$ (free $Mg^{2+}$ = 0.5 mM), pH 7.4 at 38°C (aerated with 95%$O_2$ and 5% $CO_2$).

**Mode of delivery**: The preservation solution was delivered continuously at a 'constant perfusion' head of 30 $cmH_2O$ with temperature maintained using a refrigerated water-bath. It was gently aerated with 95% $O_2$ 5% $CO_2$ taking care to avoid wide swings in pH. While it is true that cold immersion storage (4°C) is the most popular technique for long term heart preservation (4-6 hrs), over the past 10 years many studies have demonstrated the superiority of the 'constant perfusion' method for heart protection and preservation. Some of the advantages of 'continuous perfusion' over cold immersion storage are: (1) reducing the likelihood of ischaemia, anaerobic metabolism and reperfusion injury, (2) increased supply of nutritional requirements (ie. energy substrates), and (3) removal of waste products from the coronary circulation. In summary, the available published data demonstrate that continuous perfusion improves preservation of donor hearts

compared to "static" immersion cold storage. However, the present invention and methodology does not preclude the use of static storage.

**Example 1**

**Arrest time** 15 hours

**Preservation solution** (as described in Materials and Methods). Preservation temperature was 10°C.

**Reperfusion Solution**: Oxygenated Krebs Henseleit containing 10 mM glucose, 70 mM sucrose and 1 mM pyruvate. Reperfusion temperature was at 37°C.

**Results**: Table 1 summarises the effect of the new invention on the isolated rat heart after 15 hours arrest at 10°C. At 5 min the heart recovered nearly full function (87-100%).

| | Heart rate (bpm) | Systolic Press (mmHg) | Diastolic Press (mmHg) | Aortic flow (ml/min) | Coronary Flow (ml/min) |
|---|---|---|---|---|---|
| **Pre-arrest** | 257 | 124 | 69 | 36 | 13 |
| **Recovery 5 min % of control** | 224 (87%) | 132 (100%) | 69 (100%) | 32 (89%) | 13.8 (101%) |

**Example 2**

**Arrest time** 12 hours

**Preservation solution** (as described in Materials and Methods but with 90 mM sucrose, not 70 mM sucrose). Preservation temperature was 10°C.

**Reperfusion Solution:** Oxygenated Krebs Henseleit containing 10 mM glucose, 90 mM sucrose, 1.0 mM pyruvate and 1.0 mM glutathione. Reperfusion temperature was at 37°C

**Results:** Table 2 summarises the effect of the new invention on the isolated rat heart after 12hours arrest at 10°C. At 5 min the heart rate recovered 61% of control, aortic and coronary flows about 50% and developed pressures a little over 100%.

| | Heart rate (bpm) | Systolic Press (mmHg) | Diastolic Press (mmHg) | Aortic flow (ml/min) | Coronary Flow (ml/min) |
|---|---|---|---|---|---|
| Pre-arrest | 243 | 136 | 70 | 46 | 16 |
| Recovery<br>5 min<br>% of control | <br>148<br>61% | <br>142<br>105% | <br>74<br>106% | <br>25<br>54% | <br>8<br>50% |

**Example 3**

**Arrest time** 12 hours

**Preservation solution** (as described in Materials and Methods but with 90 mM sucrose and no allopurinol. Preservation temperature was 10°C.

**Reperfusion Solution:** Oxygenated Krebs Henseleit containing 10 mM glucose, 90 mM sucrose and with no allopurinol and no pyruvate. Reperfusion temperature was at 37°C.

**Results:** Table 3 summarises the effect of the new invention on the isolated rat heart after 12hours arrest at 10°C. At 5 min the heart rate recovered 73% of control, aortic flow 40%, coronary flow 86% and developed pressures 110% of control measured 12 hours earlier.

| | Heart rate (bpm) | Systolic Press (mmHg) | Diastolic Press (mmHg) | Aortic flow (ml/min) | Coronary Flow (ml/min) |
|---|---|---|---|---|---|
| Pre-arrest | 333 | 114 | 71 | 40 | 21.5 |
| Recovery<br>5 min<br>% of control | <br>243<br>73% | <br>127<br>111% | <br>78<br>110% | <br>16.2<br>40% | <br>18.4<br>86% |

**Example 4**

**Arrest time** 6 hours

**Preservation solution** (as described in Materials and Methods but with 90 mM sucrose. Preservation temperature was 10°C.

**Reperfusion Solution:** Oxygenated Krebs Henseleit containing 10 mM glucose, 90 mM sucrose and 20 uM amiloride. No allopurinol and or pyruvate. Reperfusion temperature was at 37°C

**Results:** Table 4 summarises the effect of the new invention on the isolated rat heart after 12hours arrest at 10°C. At 5 min the heart rate recovered 60% of control, aortic flow 63%, coronary flow 120% and developed pressures 93-118% of control measured 12 hours earlier.

| | **Heart rate (bpm)** | **Systolic Press (mmHg)** | **Diastolic Press (mmHg)** | **Aortic flow (ml/min)** | **Coronary Flow (ml/min)** |
|---|---|---|---|---|---|
| **Pre-arrest** | 385 | 115 | 75 | 43.5 | 17.5 |
| **Recovery 5 min % of control** | 233<br>60% | 136<br>118% | 70<br>93% | 27.5<br>63% | 21<br>120% |

**General conclusion:**

The results from four examples show that the new long-term preservation solution can preserve the rat heart for up to 15 hours with good to excellent recoveries measured at five minutes after the onset of reperfusion.

It will be understood that the invention disclosed and defined in this specification extends to all alternative combinations of two or more of the individual features mentioned or evident from the text or drawings. All of these different combinations constitute various alternative aspects of the invention.

It will also be understood that the term “comprises” (or its grammatical variants) as used in this specification is equivalent to the term “includes” and should not be taken as excluding the presence of other elements or features.

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